Efficacy and Mechanism of Action of a Calcium Channel Blocker After Global Cerebral Ischemia in Rats

James C. Grotta, MD, L. Creed Pettigrew, MD, Daniel Rosenbaum, MD, Catherine Reid, BS, Howard Rhoades, PhD, and David McCandless, PhD

Dihydropyridine calcium channel blockers such as nicardipine are under evaluation for treating acute cerebral ischemia because they may increase cerebral blood flow by causing vasodilation and because they may be cytoprotective in part by limiting production of arachidonic acid metabolites. We demonstrated in a previous study that nicardipine improves postischemic neuronal function, as measured by somatosensory evoked potentials, without reducing the extent of light-microscopic CA-1 hippocampal histologic damage. To characterize further the effect of nicardipine on global ischemic injury, we administered the drug beginning 24 hours before 30 minutes of four-vessel ischemia in Wistar rats. We then measured hippocampal ATP, phosphocreatine, and glucose contents immediately and 2 hours after ischemia, and measured learning ability (working and reference errors) on an eight-arm radial maze beginning 30 days after ischemia. To gain insight into the possible mechanism of action, we measured production of arachidonic acid metabolites (eicosanoids: TXB₂, and 6-keto-PGF₁α) and hemispheric and hippocampal cerebral blood flow by the [¹⁴C]butanol indicator fractionation technique immediately and 2 hours after ischemia. Nicardipine was associated with fewer working errors (p<0.02) but no difference in reference errors. The drug had no effect on energy metabolites, cerebral blood flow, or eicosanoids immediately after ischemia, but ATP, phosphocreatine, and cerebral blood flow all returned to normal levels significantly more rapidly during reperfusion in treated rats. Nicardipine improves behavioral, electrophysiologic, and mitochondrial function after ischemia without preventing cellular damage and improves postischemic reperfusion. The drug's positive effect appears to occur during reperfusion. (Stroke 1988;19:447–454)

Numerous studies have evaluated the effect of calcium channel blockers in animal models of cerebral ischemia, but the efficacy and mechanism of action of these drugs are still debated. Nevertheless, the rationale behind their use and experimental results to date have been sufficiently encouraging that therapeutic trials in human stroke patients are underway.

Our previous work has demonstrated that nicardipine (NC), a dihydropyridine calcium channel blocker, administered either before or immediately after ischemia improves outcome as measured by somatosensory evoked potentials (SEPs) but will not reduce the extent of histologic damage. Furthermore, we have demonstrated NC uptake into normal and ischemic brain, suggesting that biologically active quantities of the drug are available to neuronal dihydropyridine receptors.

There are two proposed mechanisms by which calcium channel blockers might ameliorate the effect of cerebral ischemia: relaxation of vascular smooth muscle, resulting in vasodilation and improved cerebral perfusion; and prevention of calcium flux into neurons, thereby limiting activation of phospholipases, proteases, and consequent membrane and protein degradation and production of damaging metabolite by-products such as free radicals. Although data are conflicting, there is a consensus that dihydropyridines increase cerebral blood flow (CBF) after ischemia in animals and humans. Our previous studies indirectly suggest that NC is also effective by the second mechanism, prevention of calcium flux into neurons, since NC improved function even when administered after CBF had returned to normal and since we demonstrated availability of NC to neuronal as well as to vascular dihydropyridine receptors. Furthermore, studies of nimodipine treatment for vasospasm after subarachnoid hemorrhage demonstrated improved outcome but no reduction of angiographic spasm, also indirectly supporting a direct neuronal protective effect of dihydropyridines.
measurements of mitochondrial function and phospholipid metabolites, which might reflect calcium-activated neuronal disruption. Measurements were performed at the end of ischemia and during reperfusion to determine if NC’s positive effect occurs as a result of blunting the ischemic insult or by correcting perturbations during reperfusion.

**Materials and Methods**

**Production of Ischemia**

Three-hundred-gram male Wistar rats were rendered ischemic for 30 minutes by a modification of the Pulsinelli method. Under chloral hydrate anesthesia, rats were ventilated using a Harvard animal respirator (model 681, Harvard Bioscience, South Natick, Massachusetts), and both vertebral arteries were permanently cauterized while the alar foramina were directly visualized. Twenty-four hours later both carotid arteries were clipped, and if necessary, cervical muscle collateral channels were occluded on one side of the neck by tightening a ligature around the cervical muscles, being careful not to obstruct the trachea. All rats were monitored for blood pressure, arterial blood gases, temperature, plasma glucose, and electroencephalogram (EEG) throughout the ischemic period. The cervical muscle ligature was applied only if the rat lost its righting reflex but some electrical activity remained. Adequate ischemia and were included for further analysis. For all studies, rats were kept ischemic by occluding the carotid arteries (and cervical collaterals if necessary) for 30 minutes. All rats were fed ad libitum before surgery, then allowed nothing by mouth except liquids until after ischemia, at which time they were allowed free access to chow and drink.

In those rats treated with NC, an Alzet osmotic pump (model 2 ML1, Alza Corporation, Palo Alto, California) was implanted subcutaneously in the suboccipital region immediately following vertebral artery surgery, 24 hours before carotid ligation. Nicardipine hydrochloride was dissolved in 3 ml sterile water with no preservatives and added to the pump to provide a continuous subcutaneous infusion of 0.05 mg/kg/hr for 96 hours. In previous studies, this dosing paradigm achieved steady-state plasma levels of 50–200 ng/ml after 24 hours and was associated with a 10% reduction in mean arterial blood pressure. Higher doses were not used because we did not want significant hypotension to complicate the interpretation of CBF and outcome measures.

**Assessment of Learning**

Ten NC-treated ischemic rats were tested on an eight-arm radial maze (each arm 86 x 10 cm) and compared with 10 untreated ischemic controls, six NC-treated sham-operated controls, and four untreated unoperated controls. The method used was adapted from Volpe et al, who validated this outcome measurement in this ischemic model. One month following ischemia, after the rats had regained their baseline weight, all rats were deprived of food until their weight reached 80% of baseline. Maze conditioning began after the first day of food deprivation and lasted 15 days. After conditioning, all rats were given one trial daily for 65 days. For each trial, the rat was placed on the center platform facing a random direction and allowed to make choices until each of five baited arms had been chosen, until 10 minutes had elapsed, or until 16 total choices had been made. The same five arms were baited for all 65 trials for any single rat. A reference error occurred when a rat chose an arm not baited with food, and a working error occurred when a rat chose the same arm twice, regardless of whether it was a correct or incorrect arm.

Three rats were chosen randomly from both the NC-treated ischemic and untreated ischemic groups at the end of 65 trials, and their brains were removed and hippocampi examined by light microscopy as previously described. The purpose of these studies was simply to be certain that equal ischemic insults occurred in both ischemic groups. Three rats were also randomly examined from the NC-treated sham-operated group. A more rigorous comparison of light microscopic changes has been published.

**Measurement of Cerebral Blood Flow**

Hemispheric and hippocampal CBF were measured by the \[^{14}C\]butanol indicator fractionation technique previously described and validated in our laboratory. A bolus of 2.5 μCi \[^{14}C\]butanol (specific activity 0.88 mCi/mmol) was injected into the vena cava coincident with the start of continuous removal of arterial blood from the tail artery through PE-50 tubing into a 250-μl Hamilton syringe using a syringe pump set at 250 μl/min. After 10 seconds of blood collection, the rat was decapitated at the same moment the withdrawal pump was turned off. The brain was rapidly removed and the hippocampus dissected in toto from one cerebral hemisphere. The hippocampus and the other entire cerebral hemisphere were dissolved separately and processed for scintillation counting. Arterial blood gases were measured at the time of CBF determination in all rats.

CBF was measured in this fashion in 10 NC-treated ischemic rats, four immediately following ischemia and six 2 hours after ischemia, and compared with equal numbers of untreated ischemic controls. In addition, CBF measurements were performed in nine untreated unoperated controls at various Pco2 concentrations to validate the sensitivity of the method.

**Enzymatic Quantification of ATP, Phosphocreatine, and Glucose**

These measurements were carried out in three NC-treated ischemic rats and compared with three untreated unoperated and three untreated ischemic rats.
All measurements were performed either at the end of 30 minutes of ischemia or after 2 hours of reperfusion following 30 minutes of ischemia.

All rats were killed by in situ freeze-fixation of the brain. Liquid nitrogen was poured into a funnel mounted on the exposed calvarium for 3 minutes. The head was then cut off and immersed in a liquid nitrogen bath. Frozen brains were dissected out of the skulls in a Wedeen cryostat (Refrigeration for Science, Island Park, New York) at -20°C. The brains were secured to brass plates with inert brain paste and freezedried for 24 hours at -40°C. The samples could then be brought to room temperature without loss of metabolites. Samples from the strata pyramidale of the CA-1 and CA-2 sectors were isolated by hand under a dissecting microscope and weighed on a quartz fiber fishpole and weighed, and stored at -80°C. Each sample consisted of forebrain dissected free of dura, venous sinuses, olfactory bulbs, cerebellum, and brainstem. The brain sections were placed in aluminum holders and freeze-dried for 2 hours or after 2 hours of reperfusion to normal within 2 hours of reperfusion in untreated ischemic rats. Previous studies in our laboratory have demonstrated rapid reduction of eicosanoid levels during reperfusion in treated and untreated rats were not compared.

The details of eicosanoid measurement have been reported. The brains were frozen using the same technique employed for the ATP measurements. The frozen brains were chiseled out of the skulls on dry ice, weighed, and stored at -80°C. Each sample consisted of forebrain dissected free of dura, venous sinuses, olfactory bulbs, cerebellum, and brainstem. The brain was homogenized, diluted with acetic acid, centrifuged, and passed through glass filter paper to remove proteinaceous debris. The remaining solution was eluted over a reverse-phase Sep-Pak C-18 column (Waters Associates, Milford, Massachusetts). The column was then washed, eluted, dried, and then redissolved in 1 ml radioimmunoassay. Quantities of TXB2 and 6-keto-PGF1α were determined as nanograms of product per gram of brain.

**Results**

**Mortality and Physiologic Variables**

Of rats rendered ischemic, 14% of all NC-treated (n = 49) and 15% of untreated (n = 47) rats died of the ischemic insult, that is, death occurred during or within 2 hours of the ischemic period (Table 1). There was also no difference in hematocrit or blood glucose between the NC-treated ischemic and untreated ischemic groups, but as expected, the average mean arterial blood pressure was lower in NC-treated ischemic rats (t = 2.49, 0.01 < p < 0.025).

**Learning**

Thirty rats were studied in four groups: untreated unoperated controls (n = 4), NC-treated sham-operated controls (n = 6), untreated ischemic controls (n = 10), and NC-treated ischemic rats (n = 10). Reference and working errors were averaged for groups of five consecutive trials beginning after 15 days of maze conditioning and lasting for 65 trials. A two-way repeated-measures analysis of variance (ANOVA) revealed that all groups demonstrated improved performance over the testing period. There was no significant difference between NC-treated ischemic rats, NC-treated sham-operated controls, and untreated unoperated controls in reference or working errors, while untreated ischemic controls made significantly more working errors than each of the other three groups [F(1,18) = 7.705, p < 0.02] (Figure 1). By the end of 50 trials, untreated ischemic controls were no longer significantly different from the other subpopulations. Untreated ischemic rats made more errors than each of the other groups (p < 0.02).
Table 2. Hemispheric and Hippocampal Cerebral Blood Flow in Nicardipine-Treated and Untreated Rats Immediately and 2 Hours After Ischemia

<table>
<thead>
<tr>
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<th>Hemispheric</th>
<th>Hippocampal</th>
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<tr>
<td>Untreated unoperated</td>
<td>6</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Immediate after 30 min ischemia</td>
<td>4</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>4</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>After 2 hr reperfusion following ischemia</td>
<td>6</td>
<td>37 ± 5</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

* t = 3.44, df = 7, 0.01 < p < 0.025 different from low Paco2.
† t = 3.76, df = 10, 0.001 < p < 0.005 different from untreated.
‡ t = 5.85, df = 10, p < 0.001 different from untreated.

Cerebral Blood Flow

Baseline hemispheric CBF in untreated unoperated controls was 118 ± 19 ml/100 g/min. There was no significant difference between baseline hemispheric and hippocampal CBF, and CBF showed the expected elevation in three controls with high Paco2 (Table 2). CBF was comparably reduced to approximately 10% of baseline levels in both untreated and NC-treated rats at the end of 30 minutes of ischemia. CBF was still depressed 2 hours after ischemia (secondary hypoperfusion) in untreated controls (compared with baseline hemispheric CBF, t = 5.24, df = 10, p < 0.001; compared with baseline hippocampal CBF, t = 4.18, df = 10, p = 0.001).

CBF was significantly higher 2 hours after ischemia in NC-treated ischemic rats than in untreated ischemic controls and not different from baseline levels (Table 2).

ATP, Phosphocreatine, and Glucose

In untreated rats, 30 minutes of ischemia resulted in reduction of ATP, PCR, and glucose in the paramedian CA-1, where ischemic damage is most severe (Table 3; Kruskal-Wallis one-way ANOVA). In the lateral CA-1 and CA-2, where histologic damage is more moderate, there was less depression of these metabolites, with significant reduction of only PCR in the lateral CA-1 and of glucose in CA-2.

At the end of the ischemic period, NC-treated rats demonstrated a trend toward higher metabolite levels than untreated ischemic controls, but this increase did not reach significance in any single region (Table 3; Kruskal-Wallis one-way ANOVA).

After 2 hours of reperfusion, ATP had returned to normal in all regions in NC-treated rats and was significantly higher in the paramedian CA-1 than in untreated ischemic controls.

Table 3. ATP, PCR, and Glucose in MCA-1, LCA-1, and CA-2 Hippocampal Regions in Untreated Unoperated, Untreated Ischemic, and Nicardipine-Treated Ischemic Rats

<table>
<thead>
<tr>
<th></th>
<th>MCA-1</th>
<th>LCA-1</th>
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<tr>
<td></td>
<td>Untreated unoperated</td>
<td>Untreated ischemic</td>
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<tr>
<td>After 30 min ischemia</td>
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</tr>
<tr>
<td>ATP</td>
<td>11.04 ± 2.57</td>
<td>4.36 ± 3.08*</td>
</tr>
<tr>
<td>PCR</td>
<td>20.03 ± 3.49</td>
<td>7.05 ± 5.32†</td>
</tr>
<tr>
<td>Glucose</td>
<td>24.58 ± 6.01</td>
<td>4.11 ± 2.42*</td>
</tr>
<tr>
<td>After 2 hr reperfusion following 30 min ischemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>11.04 ± 2.57</td>
<td>6.55 ± 1.31</td>
</tr>
<tr>
<td>PCR</td>
<td>20.03 ± 3.49</td>
<td>21.96 ± 9.01</td>
</tr>
</tbody>
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PCR: phosphocreatine; MCA-1, paramedian CA-1; LCA-1, lateral CA-1. Values are mean ± SD nmol/mg dry wt.

* t < 0.02, 0.04 different from untreated unoperated controls.
† t < 0.05 different from untreated ischemic controls.
unacceptable drop in blood pressure. Because subcutaneous infusion of 0.05 mg/kg/hr takes up to 24 hours to achieve a blood level of 6–10 ng/ml, we elected to begin the NC infusion 24 hours before ischemia for the present study since we were interested in studying the effect of the drug on CBF and metabolism in the immediate postischemic period.

The rat four-vessel ischemia model is an appropriate choice for studying the effect of agents, such as NC, which might improve CBF or ameliorate cytotoxic events following ischemia. During ischemia, CBF falls precipitously to approximately 10% of baseline, followed by a brief hyperemic phase after release of the carotid ligatures. A period of secondary hypoperfusion then lasts at least 2 hours before CBF returns to normal by 24 hours (Table 2). Despite normal reperfusion of the brain within 24 hours, progression of ischemic damage occurs histologically for at least 72 hours in hippocampal and neocortical neurons, and these histologic changes are paralleled by suppression of SEP amplitude, indicating ongoing cytotoxicity despite return of CBF to normal.

The model we used in the present study was an adaptation of that initially described by Pulsinelli and Brierley, with certain changes incorporated as a result of our experience. We found slightly higher baseline, ischemic, and postischemic CBF after 30 minutes of ischemia than initially described, though the same trends and proportional changes were found in our results. Higher CBF in our controls may be partly explained by higher PCO2 seen in our rats, a physiologic parameter that we have confirmed in this study to have the expected substantial effect on CBF.

The most striking finding in our present study was the prevention of impaired maze performance in NC-treated ischemic rats. Working errors are the most sensitive index of ischemia-induced disruption of learning in this model, and they were virtually eliminated by NC treatment. Since there was no difference between untreated nonischemic and NC-treated sham-operated rats, the drug’s positive effect was present only in ischemic rats and appears to be due to a specific amelioration of the effect of ischemia on learning ability. This finding suggests that NC reduces ischemic damage to brain regions responsible for memory, but since light microscopic hippocampal damage was equally severe in untreated ischemic and NC-treated ischemic rats in our present and previous studies, regions other than CA-1 of the hippocampus must be involved in working memory in rats. These findings and our previous demonstration that SEP amplitude is preserved in NC-treated animals 72 hours after ischemia indicate that functional outcome after ischemia in this model does not always correlate with histologic outcome as determined by hippocampal damage. This does not mean that quantitative light microscopic measurement of ischemic injury is not an important standard for assessing outcome in this and other models, but it does suggest that carefully performed behavioral and electrophysiologic measures may be more sensitive guides to therapeutic efficacy.
than morphologic changes, \(^{43}\) thereby explaining negative results in some studies relying exclusively on histologic end points.\(^{5,10,12,14}\) Our inability to distinguish between ischemic and normal (Figure 1) animals on the reference task may have been due to the relatively mild ischemic insult produced in our rats.

The period of postischemic secondary hypoperfusion was briefer, and CBF returned to normal faster, in NC-treated ischemic rats than in untreated ischemic controls. We cannot determine from the present studies if the increase in CBF was due to NC-induced arterial dilation or linked to an increase in cerebral metabolic activity indicated by increased ATP levels in NC-treated rats after 2 hours of reperfusion (see below). These data are consistent with other studies demonstrating that NC and other dihydropyridines increase CBF after ischemia in animal models in which reperfusion occurs.\(^{1,9,11}\) Negative effects of dihydropyridines on CBF have been largely confined to studies using normal nonischemic animals\(^{59}\) or models of permanent middle cerebral artery occlusion without reperfusion,\(^{6,10,18}\) in which it would not be expected that a vasodilator would be as effective.

We did not find that NC preserved neuronal metabolic activity during ischemia as determined by direct enzymatic quantification of ATP, PCR, and glucose in damaged hippocampal regions, but we did discover that the drug caused more rapid restoration of mitochondrial function in CA-1 after 2 hours of reperfusion as determined by absolute measurements of ATP and PCR.

Our ATP assay was sufficiently sensitive to detect significant postischemic regional variations within the hippocampus, which correlated with the degree of histologic injury.\(^{44}\) We found less marked depletion of ATP and PCR at the end of 30 minutes of ischemia than other investigators using this model,\(^{46}\) however, indicating that less severe ischemia may have been produced by our 30 minutes of four-vessel occlusion. To maintain a standard degree of ischemia we scrupulously excluded all rats that did not lose their righting reflex or failed to maintain a flat EEG throughout the ischemic period and any rat that developed clinical seizures during reperfusion. As described above, however, CBF levels were slightly higher in our ischemic rats than in those described by other investigators,\(^{43}\) also indicating that less severe ischemia may have been produced. Whereas we cannot completely explain our higher ischemic energy metabolite levels and whereas the standard deviation in our measurements was high and the number of observations small, we feel our results are valid for the following reasons: the metabolite levels were consistent across all groups, they showed a consistent correlation with extent of histologic injury, they showed the expected partial recovery after 2 hours of reperfusion, and they were sufficiently sensitive to detect a significant difference between treatment and control groups during reperfusion.

Another interesting finding was the supranormal level of PCR seen during reperfusion in NC-treated ischemic rats, found to a lesser degree in our untreated ischemic rats and by Pulsinelli and Duffy\(^{58}\) in their study of ischemic untreated animals. Since the PCR-creatine equilibrium is pH-dependent, PCR assays may be elevated by an alkaline cellular environment as described in ischemic neurons during reperfusion as studied by nuclear magnetic resonance spectroscopy.\(^{59}\) It is not known if this alkaline shift correlates with neuronal viability.

NC did not prevent a dramatic rise in the production of eicosanoid metabolites immediately after ischemia. It is not known if TXB\(_2\), and 6-keto-PGF\(_{1\alpha}\) measured in ischemic brain homogenates are derived from calcium-activated arachidonic acid metabolism in neurons or from platelets and vascular endothelium, but elevation of these eicosanoids can be prevented by specific enzyme inhibitors,\(^{55}\) and measurement of fatty acid metabolism has been used by other investigators as an indirect index of the effect of calcium antagonists on preventing calcium-activated phospholipid metabolism.\(^{60}\) The inability of NC to reduce the production of TXB\(_2\) or PGF\(_{1\alpha}\) therefore, may indicate that either the drug is ineffective in preventing calcium activation of neuronal arachidonic acid metabolism at the doses used in this model or that the metabolites are derived from a noncalcium-activated source.

In conclusion, in this global ischemia reperfusion model NC improves functional outcome as determined by measurement of learning ability, increases reperfusion, and helps restore mitochondrial function during reperfusion. Its positive effect appears to occur during

| Table 4. Thromboxane B\(_2\) and 6-keto-PGF\(_{1\alpha}\) in Untreated and Nicardipine-Treated Rats Immediately After 30 Minutes of Ischemia |
|----------------|----------------|----------------|----------------|----------------|
| Individual Rats | Thromboxane B\(_2\) | 6-keto-PGF\(_{1\alpha}\) |                |                |
|                 | Untreated | Treated | Untreated | Treated |
| 1               | 14.85     | 10.19   | 4.36      | 3.28       |
| 2               | 15.76     | 23.31   | 5.32      | 4.95       |
| 3               | 12.58     | 7.12    | 3.60      | 4.19       |
| 4               | 11.91     | 5.99    | 3.44      | 3.69       |
| 5               | —         | 13.36   | —         | 1.41       |
| 6               | —         | 16.80   | —         | 1.95       |
| Mean ± SD      | 13.78 ± 1.83 | 12.8 ± 5.95 | 4.18 ± 0.86 | 3.25 ± 1.23 |

Values are ng/g body wt.
reperfusion and not during ischemia. Whether the drug acts primarily by improving CBF or by preventing intraneuronal calcium-activated processes cannot be conclusively answered by this study.

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